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Pseudo-constitutivity of nitrate-responsive genes in nitrate reductase mutants

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ABSTRACT

In fungi, transcriptional activation of genes involved in NO_3^- assimilation requires the presence of an inducer (nitrate or nitrite) and low intracellular concentrations of the pathway products ammonium or glutamine. In *Aspergillus nidulans*, the two transcription factors NirA and AreA act synergistically to mediate nitrate/nitrite induction and nitrogen metabolite derepression, respectively. In all studied fungi and in plants, mutants lacking nitrate reductase (NR) activity express nitrate-metabolizing enzymes constitutively without the addition of inducer molecules. Based on their work in *A. nidulans*, Cove and Pateman proposed an "autoregulation control" model for the synthesis of nitrate metabolizing enzymes in which the functional nitrate reductase molecule would act as co-repressor in the absence and as co-inducer in the presence of nitrate. However, NR mutants could simply show "pseudo-constitutivity" due to induction by nitrate which accumulates over time in NR-deficient strains. Here we examined this possibility using strains which lack flavohemoglobins (*fhbs*), and are thus unable to generate nitrate internally, in combination with nitrate transporter mutations (*nrtA*, *nrtB*) and a GFP-labeled NirA protein. Using different combinations of genotypes we demonstrate that nitrate transporters are functional also in NR null mutants and show that the constitutive phenotype of NR mutants is not due to nitrate accumulation from intracellular sources but depends on the activity of nitrate transporters. However, these transporters are not required for nitrate signaling because addition of external nitrate (10 mM) leads to standard induction of nitrate assimilatory genes in the nitrate transporter double mutants. We finally show that NR does not regulate NirA localization and activity, and thus the autoregulation model, in which NR would act as a co-repressor of NirA in the absence of nitrate, is unlikely to be correct. Results from this study instead suggest that transporter-mediated NO_3^- accumulation in NR deficient mutants, originating from traces of nitrate in the media, is responsible for the constitutive expression of NirA-regulated genes, and the associated phenotype is thus termed "pseudo-constitutive".

1. Introduction

With few exceptions, like the yeast *Saccharomyces cerevisiae*, most fungi are able to use nitrate as an assimilatory nitrogen source and recent work showed that fungal nitrate assimilation significantly contributes to biogeochemical nitrogen cycling in nitrate-dominated agricultural soils (Gorfer et al., 2011). Bacteria, algae and plants also assimilate nitrate and in all systems NO_3^- needs to be reduced to ammonium in order to serve as nitrogen source for incorporation into amino acids. These sequential reaction steps are carried out by the enzymes nitrate reductase (NR, NO_3^- to NO_2^-) and nitrite reductase (NiR, NO_2^- to NH_4^+) (Cove, 1979). Nitrate assimilation in *Neurospora crassa* and *Aspergillus nidulans* served as early eukaryotic model systems to study adaptive enzyme formation due to the ease with which the enzymatic activity can be assayed in cell extracts (Cove and Coddington, 1965; Kinsky and McElroy, 1958). Genetic dissection of the pathway in *A. nidulans* resulted in the characterization of mutants affected in structural genes (Cove and Pateman, 1963; Pateman et al., 1967). Amongst others, NirA and one of the first eukaryotic regulatory mutations were identified using this model pathway (Cove, 1969). This work revealed that *de novo* synthesis of NR and NiR are subject to induction by nitrate or nitrite and to repression by ammonium (Cove, 1966; Kinsky, 1961). Both inducer molecules are internalized by active transport via the two nitrate permeases *nrtA* (*crnA*) and *nrtB*

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(*crnB*), also capable to transport nitrite (Brownlee and Arst, 1983; Unkles et al., 1991, 2001), whereas *nitA* encodes a specific nitrite transporter only (Unkles et al., 2011; Wang et al., 2008).

Today, the molecular basis of nitrate-responsive gene regulation in fungi is well understood. In *A. nidulans*, nitrate induction is mediated by the binuclear Zn-cluster protein NirA (Burger et al., 1991a,b; Cove, 1976) which accumulates in the nucleus in the presence of intracellular nitrate. Nuclear accumulation is the consequence of nitrate-or nitrite- mediated disruption of the interaction between the NirA nuclear export signal (NES) and the nuclear exportin KapK (Bernreiter et al., 2007). Subsequently, NirA binds to specific recognition sites in target promoters (Strauss et al., 1998). Transcriptional activation of most nitrate-responsive genes additionally requires interaction of NirA with AreA (Arst and Cove, 1973; Arst, 1982; Caddick et al., 1986; Kudla et al., 1990; Starich et al., 1998), a GATA-type transcriptional co-activator regulating genes involved in nitrogen metabolism. In the nitrate pathway AreA is required for *in vivo* DNA binding of NirA (Berger et al., 2006; Narendja et al., 2002), for recruitment of histone acetylation activities (Berger et al., 2008) and chromatin remodeling in nitrate-responsive promoters (Muro-Pastor et al., 1999, 2004). In such a way, NirA and AreA act synergistically to activate transcription of nitrate-responsive genes including the genes coding for NR (*niaD*) and NiR (*niiA*).

Cove and Pateman noted that mutants affected in NR activity constitutively synthesize NiR without the need of inducer addition and proposed an autoregulation model in which the NR holoenzyme would act as co-repressor of its own synthesis and that of NiR in the absence of nitrate or nitrite (Cove and Pateman, 1969). This hypothesis was further supported by findings that *cnx* mutants, unable to synthesize the molybdenum-containing cofactor of NR, are likewise constitutive for NiR activity (Mac Donald and Cove, 1974; Pateman et al., 1964). Interestingly, also the enzymes of the pentose phosphate pathway, later shown to be up-regulated in a NirA-dependent manner (Schinko et al., 2010), were found to be constitutively produced in *cnx* and *niaD* mutants (Hankinson and Cove, 1974). A molecular study confirmed the constitutive expression of both *niaD* and *niiA* in selected *niaD* and *cnx* mutants (Hawker et al., 1992).

However, an alternative way to explain the constitutive phenotype would be the intracellular accumulation of nitrate in NR-negative mutants from either external or internal sources. Such external trace amounts of nitrate may occur as contaminants of media components which might accumulate over time inside NR-mutant cells by nitrate transporter activity, and eventually lead to activation of NirA. In the yeast *Hansenula polymorpha* (Navarro et al., 2003) and in the algae *Chlamydomonas reinhardtii* (Llamas et al., 2002) this was shown to be the underlying mechanism of constitutive gene expression. In both organisms, constitutivity of the nitrate assimilatory genes observed in NR mutants was lost when nitrate transporters were non-functional. In *C. reinhardtii*, the authors showed also that intracellular levels of nitrate are detectable in NR mutants grown on "nitrate-free" medium. These results weaken the hypothesis that in these organisms NR itself possesses a regulatory function. However, from these studies it cannot be formally excluded that internal sources of nitrate – such as NO_2^- or NO_3^- derived from nitric oxide (NO) – artificially induce the system in cooperation with the transporters. In this alternative model nitrate transporters would additionally act as signalers, similar to what has been shown for *Arabidopsis thaliana* (Guo et al., 2003).

Although so far no clear evidence for the existence of mammalian-type classical NO synthases is available for fungi, algae and plants, NO can be generated in these organisms by the nitrate reductase enzyme itself as a by-product of the main enzymatic reaction (Besson-Bard et al., 2008; de Montaigu et al., 2010; Modolo et al., 2005; Rockel et al., 2002; Schinko et al., 2010; Wendehenne et al., 2001; Yamasaki, 2000; Yamasaki and Sakihama, 2000). Additionally, a variety of (bio)chemical pathways are known to generate NO in metabolically active cells (Nagase et al., 1997; Zweier et al., 1999). Notably, many processes have been shown to be regulated by NO in plants, e.g. stomatal closure, flowering, gravitropsim, and stress response (reviewed in Besson-Bard et al., 2008). NO is harmful to cells at higher concentrations causing proteins to become nitrosylated or nitrated impairing their proper function. Levels of NO are antagonized by spontaneous oxidation to nitrite and peroxinitrite and by enzymatic detoxification involving flavohemoglobins (*fhb*). These evolutionary conserved di-oxygenases have the ability to convert NO directly to NO_3^- and thereby efficiently remove excess NO (Gardner et al., 1998; Poole and Hughes, 2000). We have recently characterized two flavohemoglobin genes (*fhbA* and *fhbB*) in *A. nidulans* and cells lacking both enzymes show hypersensitivity to elevated environmental NO levels. FhbA was shown to be induced by nitrate in a strictly NirA-dependent manner but interestingly, and in contrast to all other known nitrate-responsive genes, *fhbA* expression does not require the function of the general nitrogen regulator AreA (Schinko et al., 2010).

To clarify whether in *A. nidulans* NR itself has a real regulatory role and if external or internal NO_3^- sources might evoke a "pseudo-constitutive" phenotype in NR loss-of-function mutants, we used mutant strains affected in nitrate transport and metabolism, and combined them with flavohemoglobin mutants lacking NO to NO_3^- conversion. Our results show that NR mutants accumulate significant levels of intracellular nitrate leading to NirA nuclear

Table 1
A. nidulans strains used in this study.

Strain name	Genotype	Reference
WT	*veA1 biA1 yA2*	Schinko et al. (2010)
ni*aD*Δ	*veA1 biA1 pyrG89 niaD*Δ *wA3*	Schinko et al. (2010)
nrtA$^-$ *nrtB*$^-$*	*veA1 biA1 pabaA1 argB2 crnA747 crnB110*	Ukles et al. (2001)
nrtA$^-$ *nrtB*$^-$	*veA1 biA1 pabaA1 argB* complemented *crnA747 crnB110*	Schinko et al. (2010)
nrtA$^-$ *nrtB*$^-$ *niaD*Δ	*veA1 biA1 pabaA1 niaD*Δ::*argB crnA747 crnB110*	Schinko et al. (2010)
*fhb*ΔΔ	*veA1 biA1 fhbA*Δ::*argB fhbB*::*argB yA2*	Schinko et al. (2010)
*fhb*ΔΔ *niaD*Δ	*veA1 biA1 fhbA*Δ::*argB fhbB*::*argB niaD*Δ *wA3*	Schinko et al. (2010)
nirA$^-$	*veA1 pabaA1 nirA637*	Schinko et al. (2010)
niiA$^-$	*veA1*, *biA1*, *niiA4*, *pyroA4*, *nkuA*Δ::*bar*	Schinko et al. (2010)
*niiA*Δ*:AFpyrG*	*veA1 nkuA*Δ::*argB niiA*Δ::AF*pyrG pyroA4 riboB2*	This study
*niaD*Δ *niiA*Δ	*veA1 biA1 niaD*Δ *niiA*Δ::AF*pyrG wA3*	This study
*nirA*Δ*:AFriboB*	*veA1 nkuA*Δ::*argB nirA*Δ::*AFriboB pyroA4 pyrG89*	This study
FLAG:nirA$_{cDNA}$*:GFP*	*veA1 nkuA*Δ::*argB* 5′UTR$_{nirA}$:AF*pyrG*:*gpdA*p M$_{atg}$:FLAG:*nirA* $_{cDNA}$:GFP:3′UTR$_{nirA}$ *pyroA4 riboB2*	This study
*niaD*Δ *FLAG:nirA*$_{cDNA}$*:GFP*	*veA1 biA1* 5′UTR$_{nirA}$:AF*pyrG*:*gpdA*p M$_{atg}$:FLAG:*nirA* $_{cDNA}$:GFP:3′UTR$_{nirA}$	This study
nrtA$^-$ *nrtB*$^-$ *FLAG:nirA*$_{cDNA}$*:GFP*	*veA1 crnA747 crnB110 biA1* 5′UTR$_{nirA}$:AF*pyrG*:*gpdA*p M$_{atg}$:FLAG:*nirA* $_{cDNA}$:GFP:3′UTR$_{nirA}$	This study
nrtA$^-$ *nrtB*$^-$ *niaD*Δ *FLAG:nirA*$_{cDNA}$*:GFP*	*veA1 crnA747 crnB110 biA1* 5′UTR$_{nirA}$:AF*pyrG*:*gpdA*p M$_{atg}$:FLAG:*nirA* $_{cDNA}$:GFP:3′UTR$_{nirA}$	This study
TNO2A7	*veA1 nkuA*Δ::*argB pyroA4 pyrG89 riboB2*	Nayak et al. (2006)

accumulation and expression of the assimilatory genes. Flavohemoglobins apparently are not required for this activity whereas nitrate permeases are. This suggests, that also in *A. nidulans* external traces of nitrate in supposedly "nitrate-free" media are accumulating in NR mutants leading to a "pseudo-constitutive" phenotype. The transporters themselves, however, do not seem to be required for the induction process when standard levels of nitrate are present in the medium.

2. Materials and methods

2.1. Strains, media and growth conditions

Genotypes of *A. nidulans* strains used in this study are given in Table 1. Minimal (MM) and complete (CM) media for *A. nidulans* were as described by Cove (1966). Supplements were added when necessary at adequate concentrations (http://www.gla.ac.uk/acad/ibls/molgen/aspergillus/supplement.html). Strains were grown on 1% glucose minimal media (GMM) supplemented according to the relevant genotypes. Liquid cultures were grown for 14 h at 37° C, 180 rpm. 3 mM L-arginine or 5 mM L-proline were used as sole nitrogen sources. For nitrate or nitrite induction 10 mM $NaNO_3$ or 10 mM $NaNO_2$ were added 15 min prior to harvesting. Mycelia were harvested by filtration through Miracloth (Merck) including a washing step with 100 ml modified and chilled MM. The washing media lacked glucose and supplements, and $MgSO_4$ was substituted by equal amounts of $MgCl_2$.

2.2. DNA and RNA manipulations

Plasmid preparation from *E. coli* strains was carried out using the Qiagen Plasmid Mini kit following the instructions of supplier. Genomic DNA extraction from *A. nidulans* was according to Lockington et al. (1985). Southern blot analysis was carried out according to Sambrook and Russell (2001). Restriction enzymes were used according to the manufacturer's instructions (Thermo Fisher Scientific). High fidelity PCR reactions were carried out using the Phusion® Flash High-Fidelity PCR Master Mix (Thermo Fisher Scientific). For conventional PCR reactions RED Taq® ReadyMix™ (Sigma Aldrich) was used. DNA fragments were purified from agarose gels using the MinElute Gel Extraction Kit (Qiagen). Total RNA was isolated from pulverized mycelia using TRIzol® reagent according to the manufacturer's instructions (Invitrogen). Northern blots were performed as described by Narendja et al. (2002), using 15 μg of total RNA per lane and membrane. ^{32}P α-dCTP labeled DNA molecules, used as gene-specific probes, were prepared using the Random Primed DNA labeling kit following the supplier's instructions (Roche Applied Science). ^{32}P α-dCTP labeled DNA probes for nitrite reductase (*niiA*), flavohemoglobin A (*fhbA*) and ribosomal 18S rRNA (18S) were used. Signal intensities were captured on a phosphoimager screen (Storm, Molecular Dynamics, Inc.) and quantified using the ImageJ software. Readout values were normalized to the values of the loading control (18S rRNA). Normalized signals were referred to the transcriptional level in the wild type strain (WT) under inducing conditions, which were arbitrarily set to 1.0. Experimental setup was repeated at least three times and representative results from these repeats are shown.

2.3. Generation of gene deletion cassettes

Gene replacement was performed by exchanging the open reading frame (ORF) or parts thereof by sequences from *Aspergillus fumigatus* encoding orotidine-5′-phosphate -decarboxylase (AF*pyrG*, Afu2g0836) or GTP-cyclohydrolase II (AF*riboB*, Afu1g13300) and the *A. nidulans* ornithine carbamoyltransferase (*argB*, ANID_04409.3), thus complementing the corresponding *A. nidulans* mutations *pyrG89*, *riboB2* and *argB2*, respectively. Details of the primer combinations used are given in Supplementary information (Supplementary Table 2).

The *nirA* deletion cassette (*nirA*Δ::AF*riboB*) was assembled using the 5′- and 3′-UTR$_{nirA}$ sequences amplified from genomic DNA using primer 1&2 and primer 3&4 and Taq polymerase. Introduced T-overhangs allowed the insertion of the fragments into pGEM-Teasy being spaced by the AF*riboB* sequence which was amplified from *A. fumigatus* genomic DNA using oligonucleotide combinations primer 5&6. Assembly of the deletion cassette was achieved in a single ligation step generating the plasmid pGEM-Teasy_*nirA*Δ::AF*riboB*.

The construction of the *niaD* deletion cassette (*niaD*Δ::*argB*) followed the same principal but replacing only part of the ORF. The 5′- and 3′- regions of the ORF were amplified using primer combinations 7&8 and 9&10. Again, a single ligation step was used to join the *argB* encoding fragment, obtained from plasmid pMS12 (FGSC, http://www.fgsc.net), together with the 5′- and 3′-ORF$_{niaD}$ sequences resulting into plasmid pGEM-Teasy_5′-ORF$_{niaD}$:*argB*:3′-ORF$_{niaD}$.

Finally, for the *niiA* deletion cassette (*niiA*Δ::AF*pyrG*), the 5′- and 3′- UTR$_{niiA}$ regions amplified using oligonucleotide combinations primer 11&12 and primer 13&14 were ligated into pGEM-Teasy creating the intermediate plasmid pGEM-Teasy_5′-UTR$_{niiA}$:3′-UTR$_{niiA}$. After the re-opening with *Sph*I the AF*pyrG* fragment (primer 15&16), obtained from plasmid p1439 (Szewczyk et al., 2006) was inserted resulting the plasmid pGEM-Teasy_5′-UTR$_{niiA}$:AF*pyrG*:3′-UTR$_{niiA}$.

All deletion cassettes were *Not*I released from the final plasmids and used for *A. nidulans* transformations.

2.4. Construction of nirA:GFP fusion cassette

The *A. nidulans* strain harboring a FLAG:*nirA* $_{cDNA}$:GFP fusion protein originally was designed for another experimental setup though was used for crossings. Since its construction has not been published elsewhere the cloning is described here. A step by step strategy was applied to assemble the chimeric cassette starting with the amplification of the 5′-UTR$_{nirA}$ from genomic DNA (primer 17&18) and the 3′-UTR$_{nirA}$ using oligonucleotides primer 19&20. The two fragments were inserted into the ApaI/PstI sites of the pGEM-Teasy vector, possessing a common KpnI restriction site. The sGFP sequence used originated from pERE-*nirA*^{c}*1*-GFP (Bernreiter et al., 2007) using primer 21&22 by which a FLAG-tag coding sequence was added at the 5′-end spaced by an NcoI site from the sGFP sequence. The KpnI/NotI construct was inserted into pGEM-Teasy_5′-UTR$_{nirA}$:3′UTR$_{nirA}$ along with a 0.65 kb *gpdA*$_p$ promoter fragment region (Vogt et al., 2005) amplified using primer 23&24. The resulting plasmid was termed pGEM-Teasy_5′-UTR$_{nirA}$:FLAG:sGFP:3′UTR$_{nirA}$,. The *nirA* cDNA sequence, amplified using primer 25&26, was inserted between the FLAG and sGFP sequence and the AF*pyrG* encoding sequence, generated with primer 27&28, was inserted upstream of the *gpdA*$_p$ promoter resulting in plasmid pGEM-Teasy_5′UTR$_{nirA}$:-AF*pyrG*:*gpdA*p Matg:FLAG:*nirA* $_{cDNA}$:GFP:3′UTR$_{nirA}$. For *A. nidulans* transformation a linear fragment of the cassette was amplified using primer 17&20. Details on plasmids generated during this work and primers used for amplifications are given in Supplementary Information (Supplementary Tables 1 and 2).

2.5. Determination of intracellular nitrate (NO_3^-) levels

The method follows the procedure described in Schinko et al. (2010), with minor modifications. Briefly, mycelia were broken by grinding in liquid nitrogen and then transferred into

pre-weighed Eppendorf tubes and extracted twice using 50% methanol (v/v). The pooled supernatant (SN) was centrifuged and directly analyzed for NO_3^- and NO_2^- concentrations. Values were normalized to the dry weight (DW) of the extracted biomass and are given as μg NO_3^- g^{-1} DW. Negative NO_3^- values are calculatory artefacts which derive from nitrite concentrations which were higher before the NO_3^- to NO_2^- reduction step than after this step (nitrite levels should be at least equally high) and are thus expressed as "not detected" (n.d.). Mean values of three independent biological experiments, including standard deviation values, were calculated.

2.6. Fluorescence microscopy

Microscopic studies were carried out as described in Berger et al. (2006) using a Leica TCS SP2 confocal laser scanning microscope. Strains were grown on cover slips for 16 h at 25 °C in GMM with 3 mM L-arginine as a sole nitrogen source. For inducing conditions 10 mM $NaNO_3$ was added to the media 2 min prior to image acquisition. Images were processed with ImageJ 1.41o and Photoshop CS software but without changing relevant characteristics of the image.

3. Results and discussion

3.1. Nitrate transporters are required for constitutive nitrate cluster expression in niaDΔ strains

An overview of the genomic arrangement of the nitrate-induced genes tested in this work is presented in Fig. 1A. The small nitrate assimilation gene cluster comprises one of the two nitrate transporters (*nrtA*) and the two reductase genes *niaD*, coding for NR and *niiA*, coding for nitrite reductase (NiR). The second nitrate transporter gene (*nrtB*) is also located on chromosome VIII but is not linked to the cluster. The nitrate-induced flavohemoglobin gene *fhbA* as well as the constitutively expressed second flavohemoglobin gene *fhbB* (Schinko et al., 2010) are neither linked to the cluster. The promoter regions of *nrtA*, *nrtB*, *fhbA* and the bidirectional promoter separating the divergently transcribed *niaD* and *niiA* ORFs, all contain binding sites for the pathway-specific activator NirA. It is well established that mutant strains lacking nitrate reductase activity constitutively transcribe genes of the nitrate assimilation cluster when grown on non-inducing media supposedly free of inducer molecules (Cove and Pateman, 1969; Hawker et al., 1992). To test if in *A. nidulans* nitrate transporters and/or flavohemoglobins are required for the constitutive expression of nitrate-responsive genes on these non-inducing media, we tested strains carrying mutations in *niaD* singly or in combination with the two nitrate transporters *nrtA* and *nrtB*, and flavohemoglobins *fhbA* and *fhbB*. Fig. 1B shows that *niiA* is highly expressed on non-inducing (NI) media containing arginine or proline as sole nitrogen (N) sources only in the *niaD*Δ strain, but not in the wild type. We used NO_2^- as inducer to exclude indirect starvation effects that could appear when nitrate would be used in *niaD*Δ strains and to have a positive induction control also for the nitrate transporter double mutants, in which nitrite is still transported by the specific nitrite transporter NitA (Wang et al., 2008). The constitutive expression level in *niaD*Δ almost reached the nitrite-induced level in the wild type strain. As expected from a strain possessing a functional nitrite transporter, induced *niiA* levels in *nrtA*$^-$ *nrtB*$^-$ double mutants are identical to wild type but expression of *niiA* on a non-inducing N-source is lost in the triple mutant strain lacking *niaD* and both transporters. However, all strains normally respond to nitrite induction by up-regulation of *niiA*.

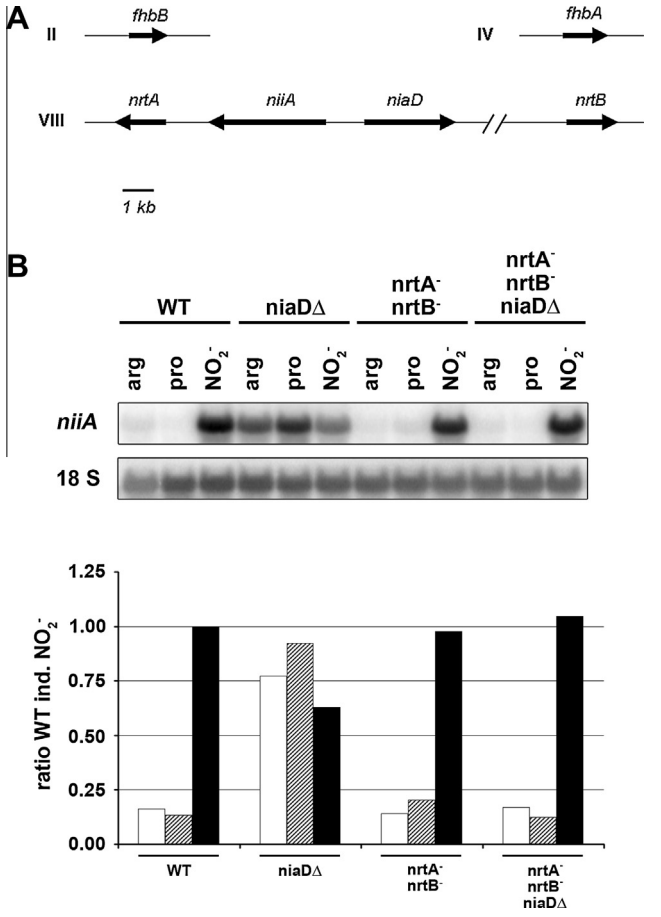

Fig. 1. (A). Overview on chromosomal positions of nitrate-responsive genes. Chromosome numbers are indicated in Roman numerals and the scale bar represents 1 kb. The nitrate cluster is located on chromosome VIII and comprises nitrate reductase (*niaD*), nitrite reductase (*niiA*) and one nitrate/nitrite transporter (*nrtA*/*crnA*). The second nitrate/nitrite transporter (*nrtB*/*crnB*) is located on the same chromosome but separated from the nitrate cluster. Genes encoding the two flavohemoglobins *fhbA* and *fhbB* are located on chromosome IV and II, respectively. Arrows indicate direction of transcription. (B). Northern blot (upper panel) and densitometric analysis (lower panel) of *niiA* steady-state levels in *A. nidulans* wild type and mutant strains lacking nitrate-related functions. Strains deficient in either nitrate reductase (*niaD*Δ), both nitrate transporters (*nrtA*$^-$ and *nrtB*$^-$) or all three genes were grown in liquid GMM for 14 h with either 3 mM L-arginine (white bars) or 5 mM L-proline (shaded bars) as sole nitrogen source. For NO_2^- induction cells (black bars) were grown on L-arginine and induced by addition of 10 mM NO_2^- for 15 min. 18S rRNA was used as loading control and reference for densitometric analysis. ^{32}P signal intensities were recorded by phosphoimaging and expression levels were calculated relative to induced wild type transcript levels which were arbitrarily set to 1.

Our observations demonstrate that nitrate permeases are not required for the signal to activate NirA but they are required to confer a constitutive phenotype on NR loss-of-function strains. Similar to the situation in the methylotrophic yeast *H. polymorpha* (Navarro et al., 2003) and the algae *C. reinhardtii* (Fernandez and Galvan, 2008) it is thus likely that also in *A. nidulans* constitutive expression of nitrate-responsive genes in *niaD*Δ strains is related to the transport of trace amounts of contaminating nitrate from the medium into *niaD*Δ mutant cells in which nitrate subsequently accumulates. These traces of nitrate, however, apparently are too low to be detected by our methods directly in the growth media or in the concentrated stock solutions and certificates of chemical analysis of the supplier of media components (e.g. L-arginine and L-proline) do not specify nitrate as contamination. The apparent Km of NrtA and NrtB are roughly 100 μM and 10 μM (Unkles et al., 2001), respectively. Thus it is likely that in NR deficient strains

nitrate levels accumulate which are sufficiently high for NirA activation.

Consistent with this interpretation, we find significantly higher levels of NO_3^- in *niaD*Δ cells grown for 14 h on L-proline or L-arginine compared to wild type cells which readily metabolize the traces of NO_3^- entering these cells (Fig. 2). In contrast, cells lacking nitrate transporters *nrtA*$^-$ and *nrtB*$^-$ show very low nitrate levels regardless whether *niaD* is present or not. These measurements are in line with the loss of constitutivity in the triple *nrtA*$^-$ *nrtB*$^-$ *niaD*Δ mutant and thus loss of constitutivity can be best explained by the lack of sufficiently high intracellular inducer levels in transporter negative strains. Based on these observations we conclude that transporter activity is necessary for inducer accumulation in a NR negative strain and that this subsequently leads to induction of NO_3^- responsive genes. Thus, it is unlikely that in *A. nidulans* NR plays a regulatory role in its own synthesis, but the source for nitrate – external or internal – is not yet clear from these results (see below).

At the molecular level this phenotype has a different basis compared to "real" constitutive *nirA*^{c} modification-of-function mutations (Cove and Pateman, 1969; Rand and Arst, 1978; Tollervey and Arst, 1981). NirA^{c}1 is now well characterized and known to carry a glycine to valine (G167V) exchange in the nuclear export signal (NES) of the protein. This amino acid substitution abolishes the interaction of the NirA-nuclear export signal (NES) with the *A. nidulans* exportin KapK. Consequently, NirA permanently accumulates in the nucleus, binds to target promoters, interacts with AreA and eventually activates target genes also under non-inducing conditions (Bernreiter et al., 2007). Because gene expression under non-inducing conditions in NR mutants is triggered by the intracellular presence of inducer we term the expression of nitrate-responsive genes in NR null mutants on "neutral" media "pseudo-constitutive".

It is surprising, however, that nitrate transporters seem to function in a *niaD*Δ background as Unkles and associates have shown that under their experimental conditions a *niaD*171 loss-of-function strain does not take up $^{13}NO_3^-$ tracer from the medium and concluded that NR activity is required for functional transport (Unkles et al., 2004). The main difference in the experimental setup was pre-loading of cells with nitrate in the case of tracer studies whereas in the experiments reported here arginine or proline were used as sole nitrogen sources. We speculate that nitrate pre-loading of *niaD* loss of function strains would result in very high intracellular nitrate concentrations since intracellular nitrate is not metabolized. These high levels of NO_3^- in the cell might block uptake of additional nitrate from the medium. In any case, data presented here formally demonstrate that nitrate reductase activity is not required for transporter function under physiological conditions.

3.2. Flavohemoglobins are not required for constitutive expression of nitrate-responsive genes

Our own experimental results as well as those obtained in *H. polymorpha* and *C. reinhardtii* NR mutants do not exclude an intracellular source of nitrate which is secreted and re-imported by transporters. We tested this hypothesis in *A. nidulans* by measuring nitrate-responsive gene expression in a triple mutant background lacking *niaD* and both flavohemoglobin genes *fhbA* and *fhbB*. We reasoned that in this strain the intracellular generation of NO_3^- at least from NO is impaired and thus pseudo-constitutivity in a *niaD*Δ background should be lost or reduced. Fig. 3A shows that this is not the case because in the *niaD*Δ strain lacking flavohemoglobin function (*fhb*ΔΔ *niaD*Δ) expression of *niiA* on non-inducing arginine is equally strong as in the *niaD*Δ single mutant control strain. These results demonstrate that flavohemoglobins are not required for pseudo-constitutivity and reinforce the view that nitrate from external sources accumulates in NR-deficient cells and subsequently induces the assimilatory genes.

Despite this clear evidence it is still puzzling why pseudo-constitutivity is not observed in *niiA*Δ strains grown on non-inducing media. In such strains inducing nitrite should accumulate due to the inability of the cells to metabolize nitrite (derived from nitrate by NR or from media contaminated by NO_2^-). However, *niiA*Δ strains behave like wild type, as observed before (Cove, 1979; Hawker et al., 1992) and seen also here for the nitrate and nitrite-responsive *fhbA* gene (Fig. 3A). In fact, we have observed equal levels of intracellular nitrite in all strains including *niiA*Δ strains on neutral media (data not shown). We neither found higher levels of intracellular nitrate in *niiA*Δ cells (Fig. 2). Nitrate, theoretically, could be formed by flavohemoglobins that would convert NO derived from spontaneous NO_2^- decomposition under acidic conditions (e.g. if excess nitrite would be transported into the vacuole). The reason why nitrate but not nitrite accumulates in cells is not known at the moment. One possibility might be

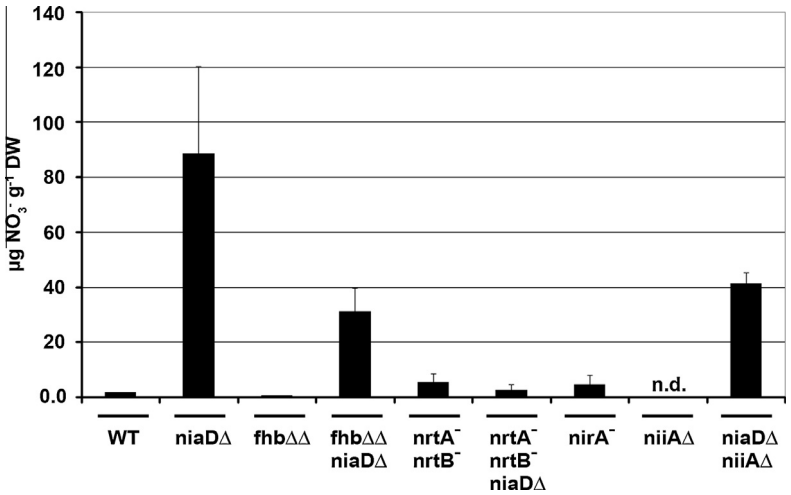

Fig. 2. Determination of intracellular nitrate levels. Intracellular nitrate concentrations were determined in *A. nidulans* wild type and different mutants strains grown for 14 h with 3 mM L-arginine as sole nitrogen source. Nitrate levels are expressed as µg NO_3^- g^{-1} dry weight (DW). Mean values and standard deviations represent data from three biological repetitions. Strain designations are wild type (WT), nitrate reductase deletion (*niaD*Δ), nitrite reductase deletion (*niiA*Δ), nitrate transporter loss-of-function mutants (*nrtA*$^-$ *nrtB*$^-$), flavohemoglobin A and B deletions (*fhb*ΔΔ) and a *nirA* loss-of-function mutant (*nirA*$^-$). n.d, not detected.

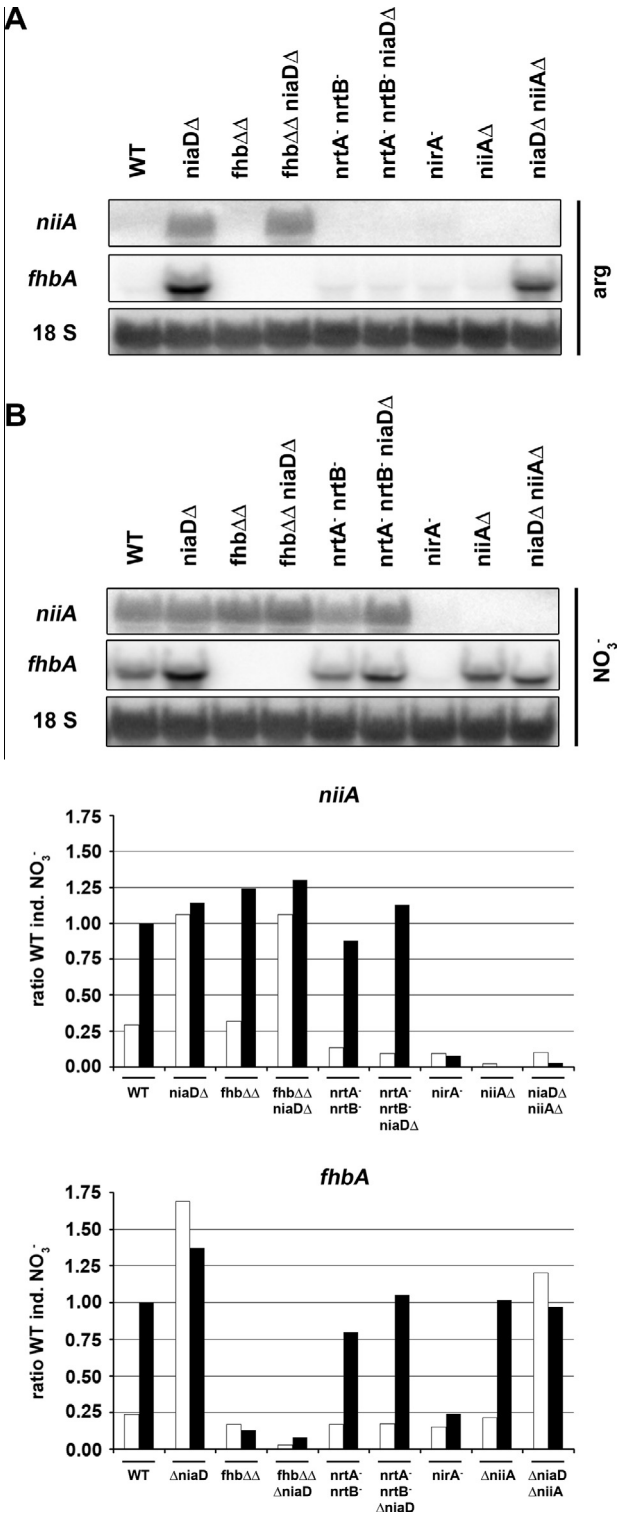

Fig. 3. Northern blot and densitometric analysis of *niiA* and *fhbA* steady-state levels in *A.nidulans* wild type and mutant strains lacking nitrate-related functions (for strain designation see Legend to Fig. 2) grown on arginine (A) or grown on arginine and subsequently induced by nitrate (B). (A). Northern blot probed for *niiA* and *fhbA* expression in strains grown on 5 mM arginine as sole N source for 14 h. (B). Northern blot (upper panel) probed for *niiA* and *fhbA* expression in strains grown on 5 mM arginine as sole N source for 14 h and induced for 15 min by addition of 10 mM nitrate. Graphs (lower panels) summarize relative expression levels of *niiA* and *fhbA* in the different strains when arginine (white bars) and arginine + nitrate (black bars) conditions are compared. 18S rRNA in each case was used as loading control and as reference for densitometric analysis. Values were calculated relative to the induced wild type level which was arbitrarily set to 1.

nitrite secretion from *niiA*Δ cells into the medium. But since the amount of NO_2^- secreted into the medium was too low to be detected by our experimental system, this possibility was difficult to verify. Another plausible explanation for lack of intracellular NO_2^- in *niiA*Δ cells is its conversion to another, non-inducing metabolite. Recently, functional dissimilatory copper-containing nitrite reductases have been identified in a number of fungi, including *Aspergillus oryzae* (Kim et al., 2009, 2010; Nakanishi et al., 2010). Although protein blast did not reveal putative orthologues of dissimilatory nitrite reductases in *A. nidulans* it cannot be excluded that proteins with more distant relation function in the same way. Although the genes encoding nitrite dissimilation activities have been shown to be strongly induced only under anaerobic conditions, a basal level of activity could be sufficient to remove the small amount of nitrite produced by nitrate reductase in *niiA*Δ strains growing on media containing trace amounts of nitrate (or nitrite). The difference between *niaD*Δ and *niiA*Δ strains in respect to pseudo-constitutivity might thus be explained by the presence of alternative nitrite reduction but the lack of alternative nitrate reduction systems.

3.3. Nitrate transporters and nitrate reductase are dispensable for nitrate signaling and NirA activation

The fact that pseudo-constitutivity is lost in the *nrtA*$^-$ *nrtB*$^-$ *niaD*Δ triple mutant could suggest that one or both nitrate transporters are involved in the signaling process for NirA-dependent transcriptional activation. *A. thaliana* contains 67 predicted nitrate transporters (De Angeli et al., 2009), among which CHL1 has been shown to display phosphorylation-dependent dual nitrate affinities and additionally carries out nitrate sensing function (Ho et al., 2009). We therefore tested our strains for transcriptional response to nitrate and nitrite induction and found that *niiA* and *fhbA* are both induced in strains carrying mutations in both nitrate transporters (Figs. 1B and 3B). Unkles and colleagues have shown that no other nitrate transporters exist in *A. nidulans* and it is thus surprising to observe an almost full transcriptional response (i.e. 80% of wild type) after 15 min of induction with 10 mM nitrate in *nrtA*$^-$ *nrtB*$^-$ strains. These results on the one hand demonstrate that the nitrate permeases do not participate in signaling towards NirA activation. On the other hand this study and previous results from our lab (Berger et al., 2008) show that intracellular nitrate is required for the NirA-dependent activation process and thus nitrate must enter *nrtA*$^-$*nrtB*$^-$ cells to some extent. This amount of nitrate must be sufficient to activate NirA but insufficient to serve as nitrogen source for growth because transporter double mutants do not grow on nitrate (10 mM) as sole N-source in plate assays (Schinko et al., 2010; Unkles et al., 2001). The mechanism of unspecific nitrate uptake is unknown but is further supported by growth tests which monitor sensitivity to the toxic NO_3^- analogue chlorate (ClO_3^-) (Suppl. Fig. 1). Chlorate is the precursor taken up and subsequently reduced to the toxic metabolite chlorite by nitrate reductase activity and thus mutants lacking NR activity (*niaD*Δ or *nirA*$^-$) are resistant to ClO_3^-. This also explains why ammonium in the growth medium (Suppl. Fig. 1, row $NH_4^+ + ClO_3^-$) protects against chlorate toxicity because nitrogen metabolite repression shuts down expression of nitrate transporters and *niaD* (Arst and Cove, 1973; Cove, 1976). In contrast to *niaD* or *nirA* mutants strains lacking *nrtA* and *nrtB* function are only moderately chlorate resistant, and high concentrations lead to strong toxicity whereas lower chlorate concentrations support residual growth of the mutant strains. This suggests that also chlorate, similar to nitrate, is taken up unspecifically when present at high concentrations. Collectively, our results suggest that the very low concentration of nitrate present in the NI medium is taken up by the highly effective specific transporters NrtA and NrtB, but unspecific alternative nitrate channels are ineffective for such low

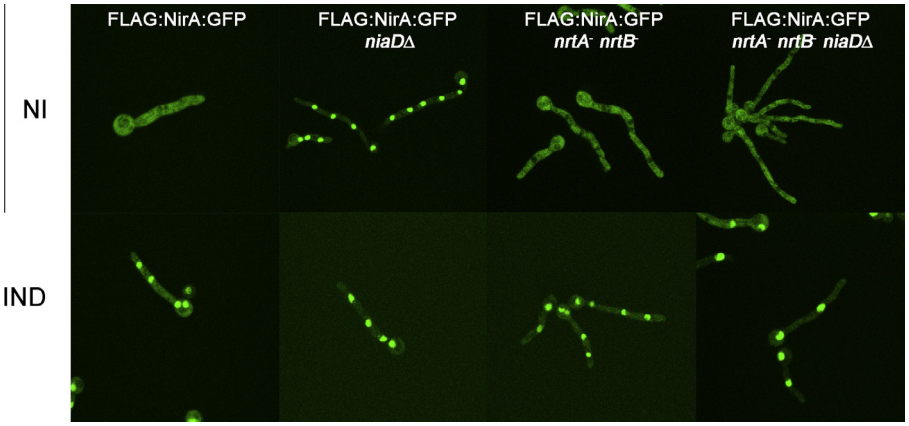

Fig. 4. Localization of the functional NirA-GFP fusion protein. Wild type and mutant strains expressing an N-terminally FLAG-tagged NirA-GFP fusion protein from the constitutive $gpdA_p$ promoter were grown at 25 °C for 16 h on 3 mM arginine (NI) or 3 mM arginine plus 10 mM nitrate (IND). In the case of nitrate induction, pictures were captured on average 2 min after the addition of inducer.

external nitrate concentrations thus leading to loss of pseudo-constitutivity of *niaD*Δ strains lacking transporter function.

To additionally clarify the relationship between nitrate permeases and the activity status of NirA, we crossed the transporter double mutant to a strain expressing a NirA-GFP fusion which functionally complements a *nirA* loss-of-function mutation (Berger et al., 2006). Panel 3 in Fig. 4 shows that in the presence of 10 mM NO_3^- NirA-GFP accumulates in the nucleus in a *nrtA*$^-$*nrtB*$^-$ background, identically to what is observed in wild type cells (first panel). Thus, small amounts of nitrate must be able to enter transporter deficient cells via an alternative route. This putative alternative nitrate channel(s), however, can only be effective when high external nitrate concentrations are present because at low nitrate concentrations, like those leading to pseudo-constitutivity in *niaD*Δ strains, they do not function (loss of pseudo-constitutivity in *nrtA*$^-$*nrtB*$^-$ *niaD*Δ triple mutants, compare Fig. 1B). This is further substantiated by the finding that NirA-GFP does not accumulate in the nucleus on the neutral nitrogen source arginine (NI) in the *nrtA*$^-$*nrtB*$^-$ *niaD*Δ triple mutant (Fig. 4, lane NI, last panel).

Using this experimental approach we were also able to finally show that nitrate reductase does not act as a co-repressor of NirA in the absence of nitrate, as suggested by the autoregulation model put forward by Cove and coworkers. NR is a cytosolic protein (Takasaki et al., 2004) and if it would scavenge NirA preventing nuclear accumulation, then NirA-GFP should be nuclear in all *niaD*Δ strains. However, this is not the case and NirA-GFP only accumulates in the nucleus under non-inducing conditions when the nitrate transporters are active. It thus is highly unlikely that NR inactivates NirA by physical interaction in the absence of nitrate and that the inducer acts to compete for the NirA-NR interaction surface thereby releasing NirA from NR inhibition. Instead, NirA seems to require only small amounts of intracellular inducer – not sufficient to promote hyphal growth – which eventually leads to disruption of the NirA–KapK interaction and subsequent NirA nuclear accumulation, specific DNA binding, interaction with AreA and finally promoting transcriptional activity of nitrate responsive genes.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.fgb.2013.02.003.

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